Physiology of toluene-degrading Pseudomonas strains under variuous conditions of nutrient limitation in chemostat culture

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SUMMARY

This thesis is concerned with the influence of the growth conditions on the physiology of bacteria capable of degrading toluene and other monoaromatic hydrocarbons. Unless stated otherwise, the experiments were performed with *Pseudomonas putida* mt-2. This strain harbours the TOL plasmid pWW0 encoding the TOL pathway, through which toluene, *m*-xylene, *p*-xylene, *m*-ethyltoluene and pseudocumene can be mineralised. We have studied the effect of environmental factors on 1) the competitive behaviour of this strain and 2) the expression levels of TOL pathway enzymes.

In order to achieve significant biodegradation of a certain organic pollutant it is essential that the bacterial strain possessing the required biodegradative abilities, can multiply and give rise to a subpopulation of sufficient size. One of the factors that determines the proliferation of a strain is its ability to compete successfully with similar strains that do not possess the biodegradation pathway involved. This competitiveness will be determined by the advantage and disadvantage respectively that are inherent with the maintenance and expression of the degradation pathway. The advantage is formed by the extra carbon and energy that the bacterial cell can derive from the oxidation of pathway substrates. The disadvantage (burden) consists of the expenditure of energy and building blocks to the maintenance (DNA replication) and expression (protein synthesis) of the catabolic pathway. Chapter 2 gives an insight into the influence of the growth conditions on the burden inherent to the maintenance and expression of the plasmid encoded TOL pathway. With the purpose of studying the growth burden independently, the cells were grown under conditions during which the TOL degradation pathway does not furnish its host with any advantage: succinate was the sole carbon and energy source (C-source) and no substrates of the TOL pathway were supplied. Under these non-selective conditions, the TOL plasmid appeared to be genetically stable: the segregation rate (frequency of loss of the TOL genes) was below 10^{-4} per generation. The cells lacking the TOL genes (TOL-minus mutants) appeared to lack a 39 kb segment harbouring the TOL genes. The growth disadvantage that the wild type strain had in comparison to those TOL-minus mutants was shown to depend strongly on the type of nutrient limitation and the growth rate. The smallest growth disadvantage of the wild type strain was found under phosphate-limitation, a condition during which the (constitutive) expression of the TOL pathway was found to be very low (see chapter 6). The growth rate disadvantage of the wild type strain under sulphate-limitation was significant, but not as large as under C-limitation, the conditions during which the (constitutive) expression was found to be relatively high. Such a derepression of a catabolic pathway under C-limitation (see also chapter 5 and 6) in the absence of an inducer is a common phenomenon. Derepression may be advantageous because it allows the immediate consumption of a transiently appearing substrate, but, as appears now, it may also be harmful for the competitiveness of the bacterial strain. The copy number of the TOL plasmid is low and is believed not to vary with the growth conditions. Therefore, the results from chapter 2 are an indication that the expression of the TOL pathway, rather than replication of the plasmid determines the degree of growth disadvantage under non-selective conditions.

In chapter 3, the influence of benzoate on the growth advantage of TOL-minus mutants was studied. Benzoate is a special C-source for *P. putida* mt-2, since the strain possesses two degradation pathways for benzoate: the chromosomally encoded *ortho* pathway and the TOL plasmid encoded *meta* pathway. In earlier studies, growth on benzoate was frequently applied to generate TOL-minus mutants. It was often assumed that benzoate increases the rate at which TOL-minus mutants are formed (the segregation rate). The results from chapter 3 indicate that the segregation rate is not increased during growth on benzoate (smaller than 10^{-4} per generation, which is similar to the frequency during growth on succinate). Mathematical analysis further indicated that the increase in TOL-minus mutants during growth on benzoate may be well explained by an approximately 15% higher growth rate in comparison to the wild type cells. The growth rate advantage of TOL-minus mutants is also manifested when succinate is present in addition to benzoate. This suggests that growth rate disadvantage is related to the expression of the *meta* pathway (mechanism unknown) and that a possible difference in the benzoate conversion rate through the *meta* or *ortho* pathway is not the main cause.

Chapter 4 describes the competition in chemostat culture between various strains that use different pathways for the degradation of toluene. The

goal was to assess how the degradation pathway influences the competitiveness of the strains under conditions of toluene- or oxygen-limitation. Both conditions may exist under field conditions. Low toluene concentrations may appear as a consequence of the tendency of toluene to partition in an organic phase (e.g. oil). Oxygen limitation may occur as a result of its low solubility and slow diffusion. Under toluene limitation, *P. mendocina* KR1, in which initial attack is by monooxygenation of the aromatic nucleus at the *para* position, outcompeted all other strains. Under oxygen-limitation, *Burkholderia cepacia* G4, which hydroxylates toluene in the *ortho* position, was the most competitive strain. *P. putida* mt-2, which metabolizes toluene via oxidation of the methyl group, was the least competitive strain under both growth conditions. The results from chapter 4 have made clear that toluene is a relatively poor substrate for the TOL pathway and that this pathway is presumably not relevant for the degradation of toluene under field conditions. This conclusion gave cause for the use of *m*-xylene rather than toluene as a selective C-source for *P. putida* mt-2 in later studies. The specific affinity of *P. putida* mt-2 for m-xylene is very high (chapter 7) and, moreover, alternative pathways for the degradation of *m*-xylene are scarce. Therefore it may be assumed that under field conditions, the degradation of *m*-xylene (in contrast to toluene) is dependent to a large degree on bacterial strains harbouring a TOL pathway.

The last three chapters of this thesis are devoted to the influence of various growth conditions on the inducibility of the TOL pathway. The expression of the TOL upper pathway was shown to be subject to strong catabolite repression. This implies that the strain has a regulatory system, that causes inducing compounds (like toluene or one of the xylenes) to express the upper pathway solely when the bacterial cells are in need of carbon or energy. Expression of the upper pathway is neither seen when cells grow at maximal rate with succinate as a C-source (chapter 5) nor when the cells grow under anabolic nutrient limitations such as sulphate- or phosphate-limitation in the presence of growth saturating concentrations of succinate (chapter 6 and 7). mRNA analysis in collaboration with the Estacion Experimental del Zaidin in Granada showed that catabolite repression was due to a blockage at the transcriptional level. Chapter 7 further describes how the inducibility of the TOL pathway may also be strongly reduced when *m*-xylene is the sole C-source when an anabolic nutrient limits growth. Under these conditions (phosphate- or sulphate- limitation), the expression of the upper pathway was reduced to 3-10% of maximal expression levels. Using the substrate depletion method, it was assessed that cells grown under an anabolic nutrient limitation have a lower conversion rate and specific affinity for *m*-xylene when compared to C-limited cells. An unexpected result of chapter 7 was the high expression level of the TOL pathway in response to *m*-xylene under oxygen limitation, both in the absence and in the presence of succinate. Oxygen limitation, however, should not be considered a desirable condition for optimizing toluene degradation; at a low oxygen concentration, the conversion rate of *m*-xylene was very low, presumably due to a low affinity for oxygen of the first enzyme of the TOL pathway, xylene monooxygenase. In the additional presence of succinate, the oxygen concentration is probably further lowered, which could explain the observed decrease of the *m*-xylene conversion rates in response to succinate.

The main conclusion of the last two chapters is that catabolite repression is not solely a laboratory artifact (manifest as diauxie during non-limited growth in batch culture) but is also likely to play a role under natural conditions of low growth rate. Therefore it may be expected that the presence of primary C-sources may significantly affect the degradation rate of xenobiotic compounds in the field.